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Xenopus laevis Egg Jelly Contains Small Proteins That Are Essential to Fertilization

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The eggs of *Xenopus laevis* are surrounded by investment layers of egg jelly that interact with the sperm immediately prior to fertilization. Components of these egg jelly layers are necessary for the fertilization of the egg by incoming sperm. Eggs which are stripped of their jelly layers are refractile to fertilization by sperm, but the addition of solubilized jelly promotes fertilization. We have shown previously that the egg jelly layers are composed of a fibrous network of glycoconjugates which loosely hold smaller diffusible components. Extracts of these diffusible components were prepared by incubation of freshly ovulated eggs in high-salt buffers for 12 h at 4°C. This diffusible component extract, when incubated with sperm, promoted the sperm's ability to fertilize dejellied eggs in a dose-dependent manner. In contrast, the high-molecular-weight "structural" glycoconjugates of jelly that remain after extraction of the diffusible components did not increase fertilization efficiency of dejellied eggs nor did nonspecific proteins, carbohydrate polymers, or organic polymers. The diffusible components, analyzed by SDS–PAGE, consisted of a mixture of proteins from 4 to 180 kDa. The protein responsible for fertilization rescue appeared to be <50 kDa and appeared to self-aggregate or to bind to larger proteins. This protein component was required during sperm binding to the egg, its action required an intact egg vitelline envelope, and its action was independent of large soluble polymers such as Ficoll. © 1999 Academic Press

Key Words: **egg jelly; fertilization; sperm–egg interaction; *Xenopus laevis*.**

INTRODUCTION

An extracellular matrix layer surrounds the eggs of most animals, and this egg covering must be penetrated and traversed by sperm before fertilization. The amphibian egg is surrounded by two distinct types of extracellular matrix, the outer jelly coat layers and an inner egg envelope referred to as the vitelline envelope. These extracellular matrix layers are known to play a number of roles during fertilization, including sperm binding, induction of the sperm acrosome reaction, polyspermy block, and protection of the developing embryo, roles that are commonly shared by egg extracellular matrices throughout the animal kingdom (Katagiri, 1987; Hedrick and Ishihara, 1991). Until recently most studies in amphibians have focused on the role of the vitelline envelope in mediating these functions with relatively little attention having been paid to the jelly layers. There has now accumulated a considerable amount of evidence that the jelly layers also play a vital role in sperm–egg interactions.

In anurans, the number of distinct jelly coats ranges from three layers in *Xenopus laevis* (Freeman, 1968; Bonnell and Chandler, 1996) to as many as six in *Rana pipiens*. These coats are synthesized in the oviduct and deposited sequentially on the egg during its travel through the oviduct (Bakos *et al.*, 1990). The jelly layers of *X. laevis* have a complex structure that has been visualized *in situ* by electron microscopy (Bonnell and Chandler, 1996). These layers are composed of fibrous, high-molecular-weight glycoconjugates, termed "structural" components because of their relative stability, and lower molecular weight glycoproteins, some of which are termed "diffusible" components because they are released from the jelly matrix into the surrounding medium during spawning (Bonnell *et al.*, 1994; Yurewicz *et al.*, 1975; Bonnell *et al.*, 1996).

Previous studies have provided evidence that amphibian egg jelly layers play an important role in sperm–egg interaction (Katagiri, 1987). One approach has been to test the fertilizability of eggs harvested from different locations in the oviduct so as to determine which jelly layers are effective for fertilization (Kambara, 1953; Applington, 1975; Katagiri, 1965; Elinson, 1973). Another approach has been

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to identify substances which can recover fertilizability of dejellied eggs. Solubilized jelly preparations have been shown to substitute for intact jelly layers in promotion of fertilization (Wolf and Hedrick, 1971; Katagiri, 1973; Elinson, 1971a,b; Stewart-Savage and Grey, 1984). For example, loss of diffusible jelly components renders *Bufo arenarum* eggs refractile to fertilization, but reintroduction of these components rescues fertilizability (Barbieri and del Pino, 1975).

In *X. laevis* evidence for the presence of such biological activities in jelly has been indirect. Wolf and Hedrick (1971) and later Stewart-Savage and Grey (1984) observed that the fertilization of jellyless eggs could be facilitated by the presence of jellied eggs or by solubilized jelly. Furthermore, in the past decade, many biologists seeking to study *Xenopus* fertilization have boosted success rates in jellyless eggs by adding "egg water," the conditioned medium obtained by soaking jellied eggs in buffer, and Ficoll during insemination (Heasman *et al.*, 1991). More recently, the egg jelly coat of *X. laevis* has been shown to contain small diffusible proteins (Bonnell *et al.*, 1996) that elicit sperm chemotaxis (Al-Anzi and Chandler, 1998). In addition, we have obtained definitive quantitative evidence that *Xenopus* egg jelly also contains small (<50 kDa) proteins that are essential for fertilization of jellyless eggs (Olson *et al.*, 1997, 1998).

In this paper we show that these proteins are diffusible jelly components which have a fertilization-promoting activity that is specific to egg jelly and which must be present at the time of insemination. We also demonstrate that these fertilization-essential proteins are lost from the jelly as an egg is stored and that the loss of egg fertilizability during storage can in part be restored by readdition of these proteins. Although, we do not yet know the exact biological mechanism by which these proteins promote fertilization, we hypothesize that they may be involved in eliciting a sperm surface reorganization that may be essential to sperm–vitelline envelope (VE) or sperm–egg binding.

MATERIALS AND METHODS

Egg and Sperm Preparation

X. laevis were purchased from NASCO (Fort Atkinson, WI) and Carolina Biological Supply (Burlington, NC) and kept on a 12-h light, 12-h dark cycle. Oviposited eggs were obtained as described by Wolf and Hedrick (1971). Briefly, ovulation was induced in females by injecting 750 IU human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO) into the dorsal lymph sac. After 8–12 h, eggs were manually stripped into dry petri dishes from the females three to four times at 2-h intervals. The eggs were then dejellied by swirling in 25 mM β-mercaptoethanol (βME) in 1.5× O-R2 buffer (124 mM NaCl, 3.75 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 1.5 mM Na_2HPO_4, 10 mM Hepes) adjusted to pH 8.5. Solubilization, monitored through a dissecting microscope, was allowed to continue until most of the egg jelly was removed, but the vitelline envelope remained completely intact. The solubilized whole egg jelly (SWEJ) was then decanted and the resulting dejellied eggs were washed in several changes of 1.5× O-R2, pH 7.5. The vitelline envelope was manually removed as needed in some experiments by incubation of dejellied eggs in 20% sucrose in 1.5× O-R2 buffer for 10 min, then manually dissecting away the loosened vitelline envelope (Stewart-Savage and Grey, 1984).

Male frogs were decapitated and their testes were removed and macerated in 2 to 3 ml of chilled 1.5× O-R2 buffer. The sperm suspension was collected and stored on ice until use. Sperm density was measured by hemocytometer and was normalized to 5×10^7 sperm/ml by centrifugation or dilution.

Preparation of Diffusible and Structural Egg Jelly Components

Eggs were manually stripped from frogs into dry petri dishes, eggs not visibly perfect were removed, then 1.5× O-R2 buffer containing a proteolytic inhibitor cocktail (200 μg/ml PMSF (Calbiochem), 15 μg/ml leupeptin (Sigma), and 15 μg/ml pepstatin A (Sigma)) was added to just cover the eggs. The eggs were then incubated for 12–16 h at 4°C to extract the diffusible fraction of the egg jelly. The diffusible fraction was then decanted. The extracted eggs were then incubated an additional 24 h at 4°C in three changes of fresh 1.5× O-R2 buffer, and the remaining egg jelly, termed the structural component, was removed from the eggs by βME solubilization. The solubilized components were then dialyzed and concentrated through a 3-kDa cutoff Spectra/Por membrane (Spectrum, Houston, TX). These procedures resulted in a yield of 4.4 μg protein/egg of diffusible components and 3 μg protein/egg of structural components.

Separation of Individual Jelly Layers

The removal of the outermost jelly layer, J3, from the innermost egg layers was carried out as previously described (Bonnell *et al.*, 1996). Briefly, J3 was grabbed with a pair of fine forceps, and a small cut was made in the J3 layer with the edge of a hypodermic needle. The small incision thus made was enlarged by grasping the edges of the incision and peeling the outer layer from the egg as if removing the skin from a grape. J3, separated cleanly from the outer edge of J2, was solubilized with βME. Any adherent pieces of J3 left clinging to J2 were removed by carefully touching the egg to a dry piece of Whatman filter paper. After removal of J3, the J1 and J2 layers together were solubilized using βME.

Fertilization Assay

Eggs, either jellied or previously dejellied, were placed in 60 × 15-mm petri dishes and covered with 5 ml of F-1 buffer (41.25 mM NaCl, 1.25 mM KCl, 0.25 mM $CaCl_2$, 0.06 mM $MgCl_2$, 0.5 mM Na_2HPO_4, 2.5 mM Hepes, with pH adjusted to 7.8). A 100-μl aliquot of sperm was then added directly over the submerged eggs, bringing the final sperm concentration to 1.0×10^6 sperm/ml in the fertilization assay medium, though sperm density was appreciably higher directly on the eggs. Dishes were allowed to incubate for 2 h, then the fertilization assay was stopped by the addition of 500 μl of formaldehyde, and fertilization was scored by counting cleavage-stage embryos (Wolf and Hedrick, 1971).

Polymers used for egg jelly substitution include Dextran T10 (Pharmacia), Ficoll 400DL (Sigma), polyvinylpyrrolidone (PVP) 30–70 kDa (Sigma), polyvinyl alcohol (PVA) 10 kDa (Sigma), bovine

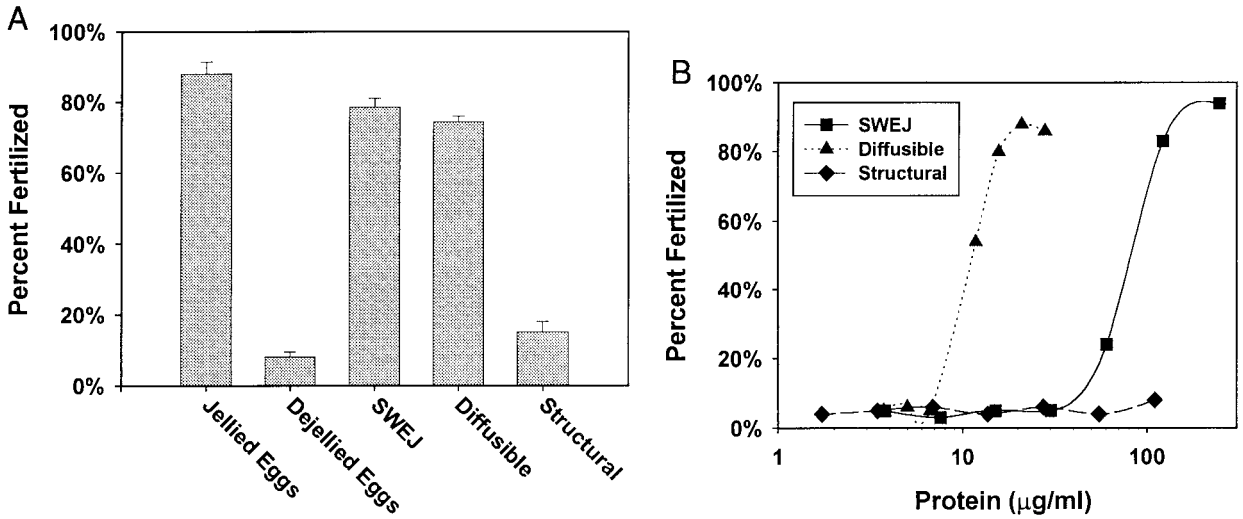

FIG. 1. (A) Diffusible jelly components restored fertilization to jellied egg levels when sperm was added to dejellied eggs. The structural fraction of egg jelly did not restore fertilization. Solubilized whole egg jelly (SWEJ) was applied at 200 μg/ml, diffusible jelly components at 25 μg/ml, and structural component at 200 μg/ml. Results are means and standard deviations for six experiments. (B) The fertilization-enhancing component was found at a much higher specific activity in the diffusible component than in solubilized whole egg jelly. A dose–response curve of fertilization-enhancing capability versus applied protein demonstrates that the diffusible component was capable of 50% rescue of fertilization at a dose of ~12 μg/ml, while seven times the amount of protein was needed for the same effect using solubilized whole egg jelly. The structural jelly components exhibited no activity. Similar results were obtained in four experiments.

cartilage powder (Sigma C-5268), and bovine serum albumin (BSA) fraction V (Calbiochem) mixed in F1 buffer.

Protein Determination

The bicinchoninic acid (BCA) kit (Pierce Chemical Co., Rockford, IL) was used to spectroscopically determine protein concentrations. BSA was used as a standard.

SDS–PAGE Separation of Egg Components

Total egg proteins, SWEJ, and diffusible and structural egg jelly components were separated on both 12.5% acrylamide and 5–20% gradient acrylamide SDS–PAGE gels with protein markers to establish relative molecular weights (Laemmli, 1970). Gels were stained for protein using Sypro orange (Molecular Probes) and visualized on a Molecular Dynamics Storm PhosphoImager.

Denaturation and Digestion of Diffusible Protein Components

Diffusible jelly components were denatured by incubation at 100°C for 45 min in F-1 buffer. Proteolytic digestion was accomplished by incubating 50 μg protein of diffusible component in a slurry of protease immobilized on beads containing either 1 unit of proteinase K–acrylamide (Sigma) or 0.5 unit of trypsin–acrylamide (Sigma) in F-1 buffer at 37°C for 45 min. The resulting digested proteins were separated from the proteases by centrifugation. For the experiments utilizing trypsin inhibitor, 200 μg trypsin inhibitor (Sigma) was added to the fertilization assay before the application of sperm (see Fig. 6).

RESULTS

The Diffusible Fraction of Egg Jelly Restores Fertilization to Dejellied Eggs

Normal jellied eggs exhibited a fertilization rate of 80 to 90% when inseminated with 1.0×10^6 sperm/ml in fertilization medium. In contrast, when jelly was removed from these eggs using β-mercaptoethanol and the eggs were washed, their fertilizability dropped precipitously to less than 10% (see Fig. 1A). To determine whether jelly components could restore fertilization competency to these eggs, separated components of egg jelly were added to dejellied eggs along with sperm. Indeed, the presence of SWEJ (200 μg/ml protein) at time of insemination boosted dejellied egg fertilizability to near control levels thereby fully substituting for the missing jelly layers. The diffusible components of *Xenopus* egg jelly (Diffusible, Fig. 1A), prepared by extracting jellied eggs with high-salt buffer (see Materials and Methods), were also capable of full restoration of fertilizability, when present at 25 μg/ml protein. In contrast, the structural components of egg jelly, remaining intact after high-salt extraction and subsequently solubilized with reductive agents, could not restore fertilization of dejellied eggs even when present at 200 μg/ml protein (Structural, Fig. 1A). A dose–response assay was performed using serial dilutions of SWEJ, diffusible, and structural jelly components (see Fig. 1B). It was found that the diffusible component (solid triangles, Fig. 1B) restored fertiliza-

tion of 50% of the dejellied eggs at a concentration of 12 μg/ml, whereas SWEJ (solid circles, Fig. 1B) needed over 90 μg/ml of protein to achieve the same result. In contrast, the structural jelly component achieved little if any restoration at concentrations up to 100 μg/ml protein. Thus, fertilization restoration activity resides largely in the diffusible jelly components while the structural components have little if any activity due to extraction of the diffusible components during preparation. This conclusion was further supported by the fact that diffusible components constituted about 15% of the total protein in SWEJ and therefore, when separated, would be expected to have a specific activity seven times higher than that of SWEJ.

Jellied Eggs Exhibit Reduced Fertilizability after Loss of Diffusible Jelly Components

Eggs which have had their diffusible egg jelly components extracted with high salt (1.5× O-R2) buffer, on visual inspection, do not appear to have lost either jelly mass or jelly solidity. The jelly does not swell due to the high salt content of the buffer, and the eggs remain fertilizable after storage in 1.5× O-R2 for up to 4 h, whereas in low-salt media the eggs hydrate and lose fertilizability competence rapidly (Wolf and Hedrick, 1971). Therefore we asked whether this loss of fertilizability was due to loss of a diffusible component, jelly hydration, or a profound physiological change in the egg due to storage.

To test for fertilizability, however, eggs had to be transferred from high-salt buffer to low-salt buffer, due to sperm motility issues (Bernardini *et al.,* 1988; Hollinger and Corton, 1980). Therefore, as shown in Fig. 2, jellied eggs were preincubated in high-salt buffer (1.5× O-R2, open symbols) or low-salt buffer (F-1, solid symbols) for times ranging from 2 to 8 h, then the eggs were transferred to fresh F-1 and inseminated. Control eggs, stored in F1 throughout preincubation, exhibited a rapid loss of fertilizability within 2 h (solid circles, Fig. 2). This loss of fertilizability could not be restored by adding SWEJ at time of insemination (solid squares, Fig. 2). In contrast, jellied eggs preincubated in high-salt buffer exhibited a much more gradual loss of fertilizability over an 8-h period (open circles, Fig. 2). In addition, the fertilization rate for these eggs could be boosted substantially by addition of either SWEJ (open squares) or diffusible jelly components (open triangles) at the time of insemination. Structural jelly components, however, were incapable of increasing fertilization rates in these eggs (open diamonds, Fig. 2). These data suggest that the loss of fertilizability in jellied eggs during storage, at minimum, is due to two separate effects. Since loss of fertilizability during storage in low-salt buffer could not be restored by jelly components, this loss is likely due to a change in jelly structure such as that brought about by hydration and swelling. On the other hand, loss of fertilizability during storage in high-salt buffer could be wholly or partially reversed by addition of diffusible jelly components, suggesting that loss of these components during short-term storage can render an egg unfertilizable. Lengthy storage (>4 h)of eggs in high-salt buffer, however, led to a progressive, irreversible loss of fertilizability the basis for which is not yet clear.

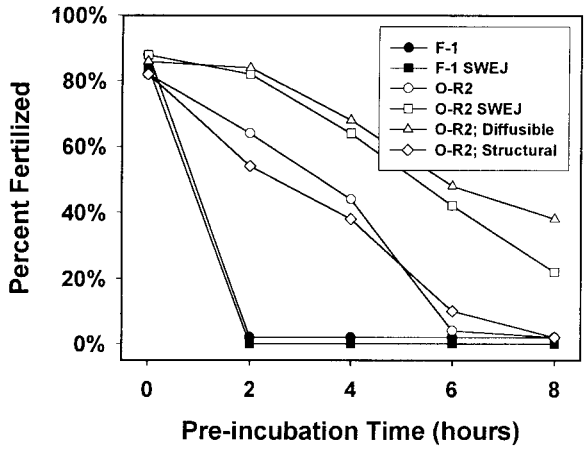

FIG. 2. Jellied eggs, incubated in either high-salt (open symbols) or low-salt (solid symbols) buffers lost fertilization capability over time. Eggs in low-salt buffer lost fertilizability rapidly and fertilizability could not be restored by jelly extracts (solid circles and squares). Eggs in high-salt buffer lost fertilizability more slowly (open circles) and this loss could in part be restored by either SWEJ (open squares) or diffusible components (open triangles), but not by structural components (open diamonds) when added to the fertilization mixture. Results are means of three experiments; standard errors have been omitted for clarity.

Fertilization-Restoration Activity Is Specific to an Egg Jelly Component

There have been reports that eggs from other anuran species (i.e., *Bufo japonicus*) can be fertilized without a jelly coat if long-chained charged polymers such as Ficoll or PVP are substituted for the missing egg jelly in the fertilization media (Katagiri, 1987). When fertilization of dejellied eggs of *Xenopus* was attempted substituting long-chain polymers such as PVP, PVA, dextran, and Ficoll in high concentrations instead of solubilized whole egg jelly, no fertilization enhancement ability was discerned (see Fig. 3). Solubilized bovine cartilage, a natural mixture of many different proteoglycans and glycoproteins, also did not enhance fertilization of dejellied eggs nor did high concentrations of BSA. These results would argue that enhancing the fertilization of dejellied eggs requires a specific component found only in egg jelly and is not a general interaction of the sperm with charged polymers, as appears to be the case in some other anuran species.

The fertilization-promoting component, though diffusible, acts in the presence of large structural glycoaminogly-

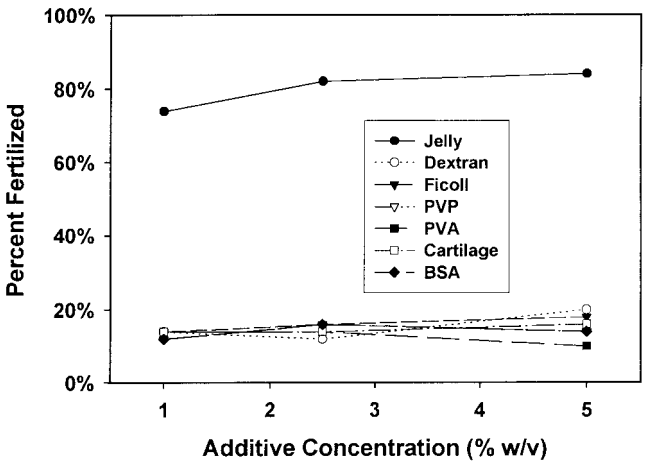

FIG. 3. Fertilization rescue activity was found only in *Xenopus* egg jelly. Jellyless eggs were inseminated in the presence of protein (BSA), carbohydrate polymers (dextran or Ficoll), organic polymers (PVP, polyvinylpyrrolidone, or PVA, polyvinyl alcohol), proteoglycans from cartilage, or *Xenopus* egg jelly and scored for fertilization. Results are representative of those obtained in two (PVP, PVA, and Ficoll) or four (jelly, dextran, albumin, and cartilage proteoglycan) similar experiments.

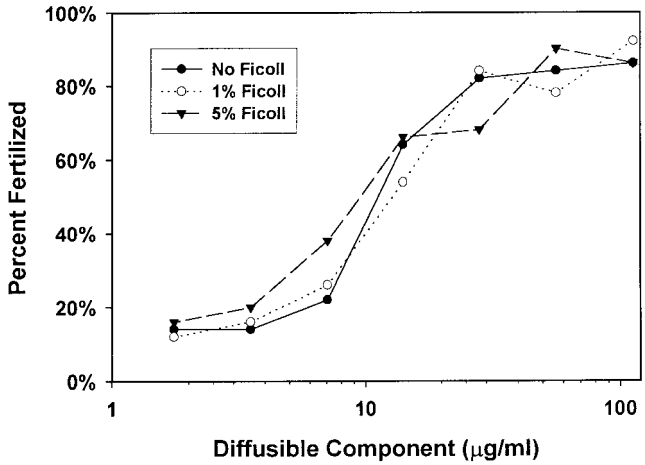

FIG. 4. Fertilization rescue activity was neither augmented or reduced by Ficoll. Jellyless eggs were inseminated in the presence or absence of *Xenopus* egg jelly diffusible components and scored for fertilization. The presence of Ficoll in the medium at either 1 or 5% w/v had no effect on the dose–response relationship for fertilization rescue by egg jelly extracts. Results are representative of three similar experiments.

cans *in vivo* due to the direct deposition of sperm on the eggs during amplexus. Might the presence of these charged polymers have a synergistic effect on enhancing fertilization? Apparently not, since the addition of 1% w/v Ficoll and 5% w/v Ficoll did not noticeably affect the concentration of soluble component needed to rescue fertilization of the dejellied eggs (see Fig. 4). These data argue that the presence of nonspecific polymers has little effect on the fertilization-enhancing properties of the soluble jelly component.

Fertilization-Restoring Activity Is Found in both Inner and Outer Jelly Layers

Since the egg jelly of *Xenopus* occurs in distinct morphological layers, and these layers are deposited sequentially on the egg, it was reasonable to ask if a specific layer of the egg jelly might contain the diffusible component needed for fertilization restoration or if any layer seems to be enriched in it. The outer layer, J3, was manually dissected away from the egg and solubilized in a mercaptan buffer, and the remaining J2 and J1 layers were also solubilized. When these two fractions were dialyzed and serially diluted, then added to the fertilization reaction of sperm and dejellied eggs, it was found that the ability to enhance fertilization was dose-dependent and that both the J3 layer and the J2–J1 layers contain substantial fertilization-promoting activity (see Fig. 5). In fact, the inner layers J1/J2 exhibited a somewhat higher specific activity (solid triangles; ED50 = 80 μg/ml) than either the outer J3 layer (solid circles; ED50 = 120 μg/ml) or the SWEJ (solid squares; ED50 = 100 μg/ml).

The Factor Responsible for Fertilization-Restoration Activity Is a Low-Molecular-Weight Protein

The active factor appeared to be heat labile and its activity was dramatically reduced after protease treatment.

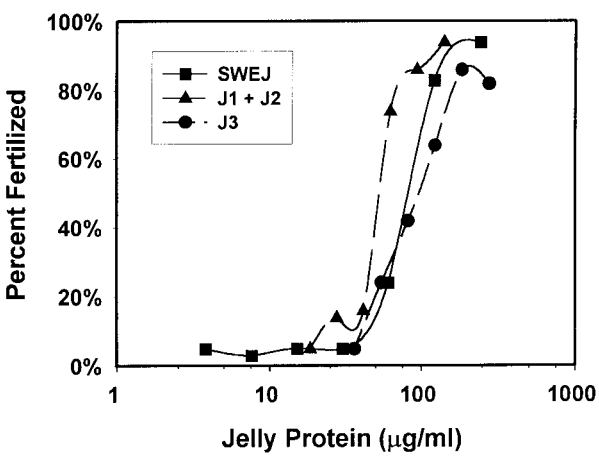

FIG. 5. Dose–response relationship for fertilization restoration activity in the inner and outer jelly layers. Activity was found in both the inner (J1 and J2) and the outer (J3) jelly layers. The results are typical of three similar experiments.

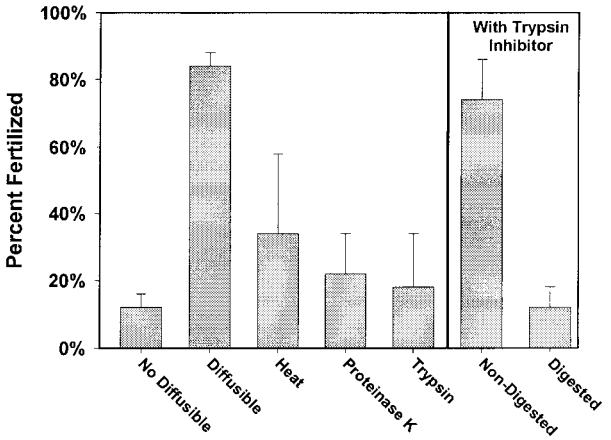

FIG. 6. The fertilization restoration activity in jelly was both heat and protease sensitive. The diffusible component was treated as described (see Materials and Methods) with heat, proteinase K, or trypsin and added to a dejellied egg fertilization assay. The panel marked "with trypsin inhibitor" defines the results of fertilization assays of trypsin-digested or undigested diffusible component in the presence of trypsin inhibitor. Results are means and standard deviations of four experiments.

A 65% loss of fertilization restoration activity was incurred by boiling the diffusible component in a low-salt buffer (F1) for 45 min (see Fig. 6, column 3), conditions under which very little protein was actually precipitated, and the boiled material exhibited no noticeable gel mobility shifts by SDS–PAGE (data not shown). Incubation of the diffusible jelly extract with proteases led to a nearly complete loss of activity. After treatment with either immobilized proteinase K or immobilized trypsin, activity of the extract was reduced by 85 or 95%, respectively (see Fig. 6, columns 4, 5, and 7). This effect was not due to residual protease activity in the fertilization assay. Both proteases, attached to beads, were removed by centrifugation before the assay. Furthermore, addition of trypsin inhibitor to the fertilization assay neither reduced nor augmented the biological activity of the diffusible components (columns 6 and 7, Fig. 6).

Initial molecular weight characterization using centrifugal filters (Centricon; Millipore, Bedford, MA) of defined pore sizes demonstrated that the majority of the activity passed through a 50-kDa molecular weight cutoff membrane (Fig. 7A), providing that the extract was filtered at its original concentration of 35 μg/ml protein. After a 10-fold concentration, the active protein no longer passed through the filter, suggesting that it underwent a concentration-dependent aggregation with either itself or a larger protein (data not shown). Diffusible and structural components of egg jelly compared by SDS–PAGE exhibited very different electrophoretic patterns (see Fig. 7B). The diffusible components (12H Diff) included proteins ranging from 4 to 180 kDa with no evidence of high-molecular-weight glycocon-

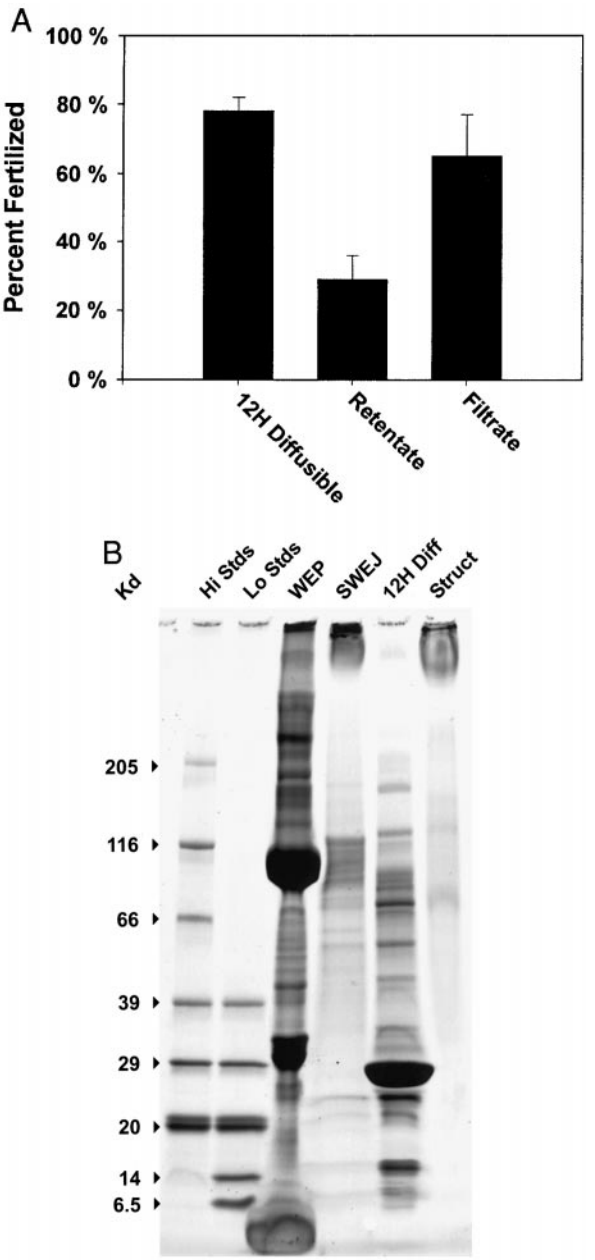

FIG. 7. (A) The fertilization-rescue activity of unconcentrated 12-h egg water passes through a 50-kDa cutoff filter. Results are means and standard errors from six experiments. (B) SDS–PAGE analysis of whole egg protein (40 μg protein, WEP), solubilized whole egg jelly (4 μg protein, SWEJ), and its diffusible (2 μg protein, 12H Diff) and structural (7 μg protein, Struct) components. Note that the diffusible components contained seven detectable bands below 30 kDa while the structural components contained only high-molecular-weight glycoconjugates. Relative mobilities were estimated using high-molecular-weight (Hi Stds) and low-molecular-weight (Lo Stds) markers from Sigma. Results are representative of three similar experiments.

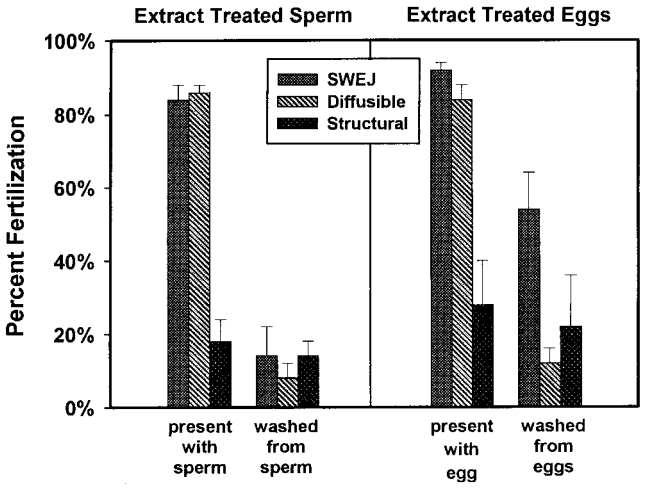

FIG. 8. Restoration of fertilization required presence of jelly components during, not before, insemination. In the panel marked "Extract-treated sperm," SWEJ or diffusible or structural jelly components were preincubated with sperm for 5 min in F-1 buffer. These components were either left in during a fertilization assay or washed off the sperm before addition to the fertilization assay. In the panel marked "Extract-treated eggs," jelly extracts were preincubated with eggs for 30 min in 1.5× OR-2 buffer, then the components were either left in or washed off prior to the fertilization assay. Results are means and standard deviations from three experiments.

jugates. Noteworthy was the presence of seven major bands below 30 kDa that are likely candidates for the fertilization rescue factor described (12H Diff, Fig. 7B). In contrast, the structural components consisted almost entirely of high-molecular-weight proteoglycans that barely entered the gel (Struct, Fig. 7B).

Fertilization Restoration Factor Must Be Present during Insemination

Sperm of many species, from mammals to sea urchins, undergo irreversible morphological and biochemical changes called "capacitation" upon contact with components of their egg's jelly coats or diffusible factors. Once sperm have encountered these "capacitative" factors, fertilization can proceed without the further participation or presence of these factors. Therefore, we sought to determine whether the fertilization-restoration factor present in *X. laevis* egg jelly is a capacitative factor or, on the other hand, whether its presence is obligatory at the time of fertilization. Sperm were pretreated with 200 μg/ml SWEJ, 25 μg/ml diffusible component, or 200 μg/ml structural component, the jelly components were washed away, and the washed sperm were then used to inseminate dejellied eggs (see Fig. 8, left, second set of bars). In each case less than 15% of the eggs were fertilized. Yet, if these components were *not* washed away before the sperm were added to dejellied eggs, the SWEJ and diffusible component raised the level of fertilization to that seen in jellied eggs (80–90%) under the same assay conditions (Fig. 8, first set of bars). This demonstrates that diffusible jelly component must be present at the moment of fertilization and that this component does not act as a capacitative agent on *Xenopus* sperm.

A similar series of experiments was carried out in which eggs were pretreated with jelly components and the components washed away prior to insemination (see Fig. 8, right, second set of bars). Again, pretreatment with either the diffusible or the structural components separately did not prime the eggs for fertilization. In contrast, when eggs were pretreated with SWEJ and the SWEJ was washed away before insemination, 55% of the washed dejellied eggs were fertilized. This finding would seem to indicate that SWEJ may "reassemble" to a limited extent on the surface of a dejellied egg, recreating enough of a jelly layer such that the components necessary for fertilization (and lost during the jelly removal) are restored. Evidently, this reassembly step did not occur with only the diffusible component present, arguing that the structural component plays a critical role in jelly assembly. Indeed, as would be expected from the above argument, preincubation of dejellied eggs with both structural and diffusible components simultaneously followed by washing and insemination did raise the level of fertilization to 45% (data not shown). The fact that this percentage was somewhat below that for SWEJ (55%) could be due to a loss of viability of the structural components during the long incubations necessary to isolate them or to a change in the ratio of diffusible to structural components from that naturally seen in SWEJ.

An Intact Vitelline Envelope Is Required for Optimal Fertilization-Restoration Activity

An intact vitelline envelope is required for these jelly components to fully restore fertilizability. Mechanical removal of the vitelline envelope from dejellied eggs (light bars, Fig. 9), resulted in *both* diffusible and structural jelly components being unable to restore fertilizability. However, SWEJ and an admixture of both diffusible and structural jelly components were still able to partially restore fertilizability, albeit to only 34 and 16%, respectively, well below the 75% fertilization rate seen in vitelline envelope-intact, dejellied eggs treated with either SWEJ or diffusible components (dark bars, Fig. 9).

DISCUSSION

Fertilization in amphibians is a mechanistic "blind date." The sperm and the egg have never previously met, but must interact with one another and hopefully find each other's presence mutually stimulating. Before the sperm and the egg initiate intimate contact and fuse, the sperm must first

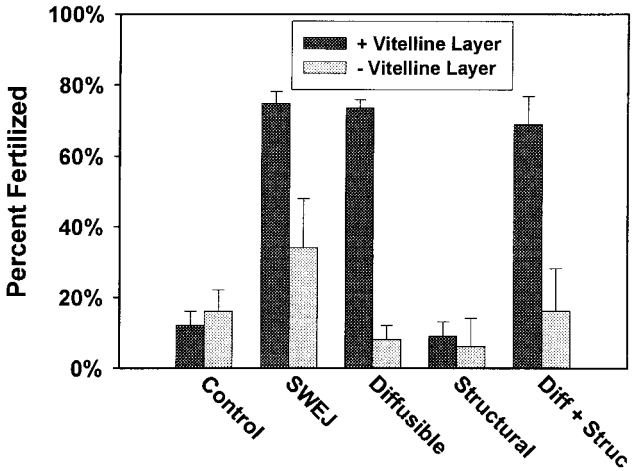

FIG. 9. Complete restoration of fertilization in jellyless eggs required the vitelline envelope. Neither the diffusible components nor the structural components alone could restore fertilizability to dejellied *Xenopus* eggs which had their VE removed. However, fertilizability of VE-less eggs could be partially restored if both components were present simultaneously. Results are means and standard deviations from three experiments.

traverse the jelly layers and bind to the surface of the egg (Hedrick and Nishihara, 1991; Omata and Katagiri, 1996). The egg jelly layers play an essential role in the mediation of this fusion event since the presence of egg jelly is necessary for fertilization in many amphibian species. These eggs when stripped of their jelly become refractile to fertilization. Readdition of solubilized jelly restores the ability of these eggs to be fertilized (Katagiri, 1965, 1974; Shivers and James, 1970; Elinson, 1971b; Wolf and Hedrick, 1971; Barbieri and del Pino, 1975; Ishihara *et al.*, 1984; Stewart-Savage and Grey, 1984). However, the macromolecular components of jelly that restore fertilization have never been fully characterized.

In this study, we have employed insemination of dejellied eggs as a powerful approach to dissecting the actual role of the egg jelly in fertilization. Here, we have demonstrated in *X. laevis* that one or more diffusible jelly proteins (as opposed to structural jelly proteins) are essential for fertilization. Furthermore, we have demonstrated that this activity is specific to egg jelly and is not found in unrelated proteins such as serum albumin or unrelated carbohydrate polymers such as Ficoll or dextran. The activity appears to reside in a small, heat-labile protein that is found in both inner and outer jelly layers. In contrast, the high-molecular-weight proteoglycans that make up the superstructure of the jelly layers appear to be inactive except for what possibly is a small residual of lower molecular weight proteins trapped in the structure.

Indeed, from these observations it is clear that *X. laevis* egg jelly is a favorable system in which to study fertilization restoration activity. Studies in other species confirm the fact that jelly is required for fertilization but have the disadvantage that even from a small sampling of species, the biological activity of jelly components is variable as to whether it is diffusible or structural and in some cases can even be replaced by synthetic organic polymers. For example, the diffusible component of the jelly of *B. japonicus* (Katagiri, 1973) and *B. arenarum* (Barbieri and Oterino, 1972) can restore the ability of sperm to fertilize dejellied eggs of the homologous species, but the diffusible component of *R. pipiens* cannot (Elinson, 1971b). *R. pipiens* requires both diffusible and structural components for sperm to fertilize dejellied eggs. Similarly, dejellied *B. japonicus* eggs can be fertilized by sperm in the presence of diffusible egg jelly components but also can be fertilized by sperm in the presence of synthetic polymers such as PVP, dextran, or Ficoll (Katagiri, 1973, 1965, 1974).

In contrast, in *X. laevis*, only small diffusible proteins, less than 50 kDa but larger than 3 kDa in size, are biologically active and in our hands this activity cannot be mimicked by either PVP or Ficoll (see Fig. 3). Currently, we are in the process of purifying these proteins using molecular sieve chromatography. Preliminary results suggest that the fertilization promotion factor is not identical to the sperm chemotaxis factor from *Xenopus* egg jelly that we have recently reported (Al-Anzi and Chandler, 1998). Although both proteins are diffusible jelly proteins of low molecular weight, the two activities differ in their heat stability and can be separated from each other by molecular sieve chromatography (J. H. Olson, preliminary studies). As demonstrated by SDS–PAGE in Fig. 7B, the diffusible jelly proteins obtained by 12-h, high-salt extraction are about 18 in number, and of these, 7 are less than 30 kDa and represent the likely candidates for fertilization-promotion activity. Of particular note in this regard are 3 major bands at 29–26, 24, and 16 kDa. Two complications are that some of these small proteins vary in occurrence from one egg batch to another and that others may be biologically active fragments of larger proteins which have been cleaved by low levels of protease activity. In this study we have attempted to minimize the latter problem by using a cocktail of protease inhibitors during extraction. Despite these complications, the current goal of our laboratory is to isolate the active protein(s) and to determine the exact mechanisms by which they act on sperm and eggs.

In the present study, we have demonstrated that these proteins are essential not only to fertilization of dejellied eggs but also to fertilization of jellied eggs. Jellied eggs incubated in high-salt buffers for up to 6 h show a decline of fertilizability that can be completely reversed by readdition of diffusible jelly proteins (see Fig. 2). Loss of fertilizability under these conditions would appear to be due not to loss of jelly structural integrity but rather to loss of small, fertilization-essential proteins into the medium. In addition, it is clear that these fertilization-essential proteins

must act during or just seconds before sperm–egg interaction since preincubation of diffusible components with either sperm or eggs *prior to* fertilization has no effect (Fig. 8). This finding is consistent with similar observations in other amphibian species (Elinson, 1971b; Barbieri and Oterino, 1972).

One possibility is that these jelly proteins are required for sperm–egg binding. In *X. laevis*, sperm first bind to the vitelline envelope, a binding that appears to be mediated by gp69 (Tian *et al.*, 1997a). Since the vitelline envelope is required for diffusible jelly components to exert their fertilization promotion activity (see Fig. 9) these jelly factors may be required during sperm–VE binding itself or for an event that prepares sperm for VE binding. Indeed, studies which have sought to quantitate sperm–VE binding have routinely exposed sperm to jelly extracts before or during binding (Tian *et al.*, 1997a,b) and investigators attempting to obtain fertilization rates that are as high as possible recommend using jelly extracts (Heasman *et al.*, 1991). Alternatively, jelly factors may influence a downstream step such as sperm–egg plasma membrane binding. Such an alternative must be considered in light of the fact that a recent study by Lindsay and Hedrick (1998) was unable to demonstrate an effect of egg water on binding of sperm to isolated *Xenopus* vitelline envelopes.

Although a classical acrosome reaction has not yet been detected in *Xenopus* sperm, such a reaction has been demonstrated in *B. japonicus* (Katagiri, 1987). This leads many biologists to believe that *Xenopus* sperm do undergo an acrosomal reaction but that it does not involve the larger scale structural changes seen in other species. Rather, it may be that in *Xenopus* sperm the acrosome reaction consists of a molecular level reorganization of the sperm surface that is required either for sperm–VE binding or sperm–egg binding. One enticing observation is that *Xenopus* sperm appear to have on their surface a metalloprotease–disintegrin protein that plays a role in sperm–egg binding and which may turn out to be a homologue of fertilizin α/β in hamster sperm. Indeed, Shilling *et al.* (1996) found that disintegrin-like peptides block sperm–egg binding in *X. laevis* and it has recently been found that antibodies to the disintegrin family of proteins will bind to epitopes at the tip of *Xenopus* sperm (J. H. Olson and D. E. Chandler, preliminary observations). Preliminary observations, in fact, suggest that exposure of these epitopes on the sperm surface may be triggered by exposure to diffusible egg jelly proteins. Determining whether this event is in fact mediated by the fertilization promotion proteins described in this study is a current goal in our laboratory.

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